

U.S. Application No.

International Application No.
PCT/EP00/07763

JG10 Recd EP/PTD 19 FEB 2002
10/069433
Attorney Docket No.
WWELL61.001APC

Date: February 19, 2002

Page 1

**TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 USC 371**

International Application No.: PCT/EP00/07763
International Filing Date: August 10, 2000
Priority Date Claimed: August 19, 1999
Title of Invention: REFOLDING OF MEMBRANE PROTEINS
Applicant(s) for DO/EO/US: Kiefer, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.
2. () This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.
3. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).
4. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. (X) A copy of the International Application as filed (35 USC 371(c)(2))
 - a) () is transmitted herewith (required only if not transmitted by the International Bureau).
 - b) (X) has been transmitted by the International Bureau.
 - c) () a copy of Form PCT/1B/308 is enclosed.
 - d) () is not required, as the application was filed in the United States Receiving Office (RO/US).
6. (X) A translation of the International Application into English (35 USC 371(c)(2)).
7. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))
 - a) () are transmitted herewith (required only if not transmitted by the International Bureau).
 - b) () have been transmitted by the International Bureau.
 - c) () have not been made; however, the time limit for making such amendments has NOT expired.
 - d) (X) have not been made and will not be made.
8. () A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).
9. () An oath or declaration of the inventor(s) (35 USC 371(c)(4)).
10. (X) A copy of the International Preliminary Examination Report with any annexes thereto, such as any amendments made under PCT Article 34.
11. () A translation of the annexes, such as any amendments made under PCT Article 34, to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

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|-----|----------------------------------|---|
| 12. | <input type="radio"/> | An Information Disclosure Statement under 37 CFR 1.97 and 1.98. |
| 13. | <input type="radio"/> | An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. |
| 14. | <input checked="" type="radio"/> | A FIRST preliminary amendment. |
| | <input type="radio"/> | A SECOND or SUBSEQUENT preliminary amendment. |
| 15. | <input type="radio"/> | A substitute specification. |
| 16. | <input type="radio"/> | A power of attorney and/or address letter. |
| 17. | <input checked="" type="radio"/> | International Application as published. |
| 18. | <input type="radio"/> | The present application qualifies for small entity status under 37 C.F.R. § 1.27. |
| 19. | <input checked="" type="radio"/> | Other Items or information: <ol style="list-style-type: none"> 1. International Search Report 2. Abstract |
| 20. | <input checked="" type="radio"/> | A return prepaid postcard. |
| 21. | <input checked="" type="radio"/> | The following fees are submitted: |

				FEES
BASIC FEE				\$890
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total Claims	19 - 20 =	0 ×	\$18	\$0
Independent Claims	1 - 3 =	0 ×	\$84	\$0
Multiple dependent claims(s) (if applicable)			\$280	\$0
TOTAL OF ABOVE CALCULATIONS				\$890
Reduction by 1/2 for filing by small entity (if applicable). Verified Small Entity statement must also be filed. (NOTE 37 CFR 1.9, 1.27, 1.28)				\$
TOTAL NATIONAL FEE				\$890
TOTAL FEES ENCLOSED				\$890

22. (X) The fee for later submission of the signed oath or declaration set forth in 37 CFR 1.492(e) will be paid upon submission of the declaration.
23. (X) A check in the amount of \$890 to cover the above fees is enclosed.

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24. ☐ Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 per property.
25. ☒ The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:


Mark R. Benedict
Reg. No. 44,531
Customer No. 20,995

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WWELL61.001APC

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Kiefer, et al.)	Group Art Unit: Unknown
)	
Int'l Appl. No. :		PCT/EP00/07763)	
)	
Int'l Filing Date:		August 10, 2000)	
)	
For	:	REFOLDING OF MEMBRANE)	
		PROTEINS)	
)	
Examiner	:	Unknown)	
)	

PRELIMINARY AMENDMENT

United States Patent and Trademark Office
PO Box 2327
Arlington, VA 22202

Dear Sir:

Preliminary to examination on the merits, please amend the above-captioned U.S. National Phase application as follows:

IN THE SPECIFICATION:

On page 1 of the English language translation of the Specification, immediately after the Title of the Invention, please insert:

This is the U.S. National Phase under 35 U.S.C. §371 of International Application PCT/EP00/07763, filed August 10, 2000, which claims priority to German Application 19939246.3, filed August 19, 1999.

On page 1 of the English language translation of the Specification immediately before the first paragraph which begins, "The present invention...", please insert:

Field of the Invention

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On page 1 immediately before the paragraph which begins, “For membrane proteins,” please insert:

Background of the Invention

On page 5 immediately after the second full paragraph, which ends “...yield is low and the method is poorly reproducible.”, please insert:

Summary of the Invention

On page 6 immediately before the second full paragraph, which begins, “The inventors of the submitted application...”, please insert:

Detailed Description of the Preferred Embodiment

On page 15, please delete the word “Claims” and substitute therefor:
WHAT IS CLAIMED IS:

On page 15, immediately after the paragraph, which ends: “...the successful refolding is shown.”, please insert page break.

IN THE CLAIMS:

Please, cancel the Claims 15 and 16 without prejudice.

Please, amend the Claims as follows:

1. **(Amended)** A method for production of proteins folded into their native or active structure, said proteins being from the family of G-protein-coupled receptors, comprising:
 - providing said protein solubilized in a first detergent, and
 - exchanging said first detergent for a second detergent, to induce folding of said protein in its native or active form, wherein said second detergent is selected from the group consisting of:
 - alkylglycosides, comprising unbranched, branched and cyclic C5-C12 alkyl chain, and glycoside, comprising monosaccharides and disaccharides; and
 - alkyl-phosphorylcholine with chain length of C10-C16.
2. **(Amended)** The method of Claim 1, wherein said second detergent is provided in a folding buffer with mixed lipid/detergent micelles.
3. **(Amended)** The method of Claim 2, wherein said folding buffer contains said second detergent and phospholipid from a natural source.

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4. **(Amended)** The method of Claim 1, wherein said exchange of detergents is done by a dialysis- or ultrafiltration method.
5. **(Amended)** The method of Claim 1, wherein said exchange of detergents is carried out via a chromatographic method.
6. **(Amended)** The method of Claim 1, wherein said exchange of detergents is carried out by diluting said solubilized protein in a buffer which contains said second detergent.
7. **(Amended)** The method of Claim 1, wherein after said exchange of detergents at least one conserved disulfide bridge is formed in said protein.
8. **(Amended)** The method of Claim 1, wherein said folded protein is incorporated in proteoliposomes.
9. **(Amended)** The method of Claim 1, wherein said protein is produced in form of inclusion bodies in a cell line transformed with an expression vector which carries a gene coding for said protein.
10. **(Amended)** The method of Claim 1, wherein said protein is part of a fusion protein and is cleaved off from said fusion protein.
11. **(Amended)** The method of Claim 9, wherein said inclusion bodies are purified and, by adding said first detergent, solubilized.
12. **(Amended)** The method of Claim 1, wherein said first detergent is selected from the group N-Lauroylsarcosine, dodecylsulfate, other charged detergents or urea or guanidiniumchloride in combination with charged or uncharged detergents.
13. **(Amended)** The method of Claim 1, wherein said second detergent has a concentration that is above its critical miceller concentration.
14. **(Amended)** The method of Claim 1, wherein said second detergent is alkyl-phosphorylcholine with a chain length of C10-C16.

Please, add new Claims:

17. **(New)** The method of Claim 3, wherein said phospholipid is a lipid extract of tissue in which said protein occurs naturally.
18. **(New)** The method of Claim 7, where the disulfide bridge is formed by adding a mixture of oxidized and reduced glutathione.
19. **(New)** The method of Claim 11, wherein said second detergent is alkyl-phosphorylcholine with a chain length of C10-C16.

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20. (New) The method of Claim 12, wherein said second detergent is alkyl-phosphorylcholine with a chain length of C10-C16.
21. (New) The method of Claim 13, wherein said second detergent is alkyl-phosphorylcholine with a chain length of C10-C16.

REMARKS

Claims 15, and 16 have been cancelled without prejudice. New Claims 17-21 have been added. Claims 1-14 have been amended to more precisely claim the invention according to conventional practice before the United States Patent and Trademark Office. Support for new Claims 17-21 can be found in the original Claims. As a result Claims 1-14, and 17-21 are presented for examination. No new matter is being added herewith. The changes made to the Specification and the Claims by the current amendment, including insertions and deletions, are shown on attached sheets entitled **VERSION WITH MARKINGS TO SHOW CHANGES MADE**, which follow the signature page of this amendment.

Should there be any questions concerning this application, the Examiner is respectfully invited to contact the undersigned attorney at the telephone appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 2/18/02

By: Mark R. Benedict
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

1. **(Amended)** A method for production of proteins folded into their native or active structure, said proteins being from the [group comprising membrane proteins, in particular receptors, preferably from the] family of G-protein-coupled receptors, [as well as partial sequences, homologous sequences, mutated sequences and derived sequences of membrane proteins and receptors, with] comprising the steps:

providing [of] said protein solubilized in a first detergent, and

[Exchange of] exchanging said first detergent for a second detergent, [which induces] to induce folding of [the] said protein into its native or active form, [characterized in that] wherein said second detergent is selected from the group consisting of:

[Alkyl-N,N-dimethylglycine (alkyl = C8-C16)]

Alkylglycosides [(alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides)], comprising unbranched, branched and cyclic C5-C12 alkyl chain, and glycoside, comprising monosaccharides and disaccharides; and

[Saccharide fatty acid ester (e.g. sucrosemonododecanoate)]

[Alkylthioglycosides (alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides with S- instead of O-glycosidic bond)]

[Bile acids (cholate, deoxycholate) and derivatives (e.g. CHAPS, CHAPSO)]

[Glucamides (MEGA-8 to -10, HEGA)]

[Lecithins and lysolecithins (e.g. DHPC, C12-lysolecithin)]

alkyl-phosphorylcholine [(alkyl = C10 - C16)] with chain length of C10-C16.

2. **(Amended)** The method of Claim 1, [characterized in that] wherein said second detergent is provided in a folding buffer with mixed lipid/detergent micelles.

3. (Amended) The method of Claim 2, [characterized in that] wherein said folding buffer contains said second detergent and phospholipid from a natural source [, preferably a lipid extract of tissue in which said protein occurs naturally].
4. (Amended) The method of Claim 1, [characterized in that] wherein said exchange of detergents is done by a dialysis- or ultrafiltration method.
5. (Amended) The method of Claim 1, [characterized in that] wherein said exchange of detergents is carried out via a chromatographic method.
6. (Amended) The method of Claim 1, [characterized in that] wherein said exchange of detergents is carried out by diluting said solubilized protein in a buffer which contains said second detergent.
7. (Amended) The method of [any of claims 1 to 6] Claim 1, [characterized in that] wherein after said exchange of detergents at least one conserved disulfide bridge is formed in said protein [, preferably by adding a mixture of oxidized and reduced glutathione].
8. (Amended) The method of [any of claims 1 to 7] Claim 1, [characterized in that] wherein said folded protein is incorporated in proteoliposomes.
9. (Amended) The method of [any of claims 1 to 8] Claim 1, [characterized in that] wherein said protein is produced in form of inclusion bodies in a cell line transformed with an expression vector which carries a gene coding for said protein.
10. (Amended) The method of [any of claims 1 to 9] Claim 1, [characterized in that] wherein said protein is part of a fusion protein and [, before or after said exchange of detergents,] is cleaved off from said fusion protein.
11. (Amended) The method of Claim 9, [characterized in that] wherein said inclusion bodies are purified and, by adding said first detergent, solubilized.
12. (Amended) The method of [any of claims 1 to 11] Claim 1, [characterized in that] wherein said first detergent is selected from the group: N-Lauroylsarcosine, dodecylsulfate, other charged detergents or urea or guanidiniumchloride in combination with charged or uncharged detergents.
13. (Amended) The method of [any of claims 1 to 12] Claim 1, [characterized in that] wherein said second detergent has a concentration that is [used] above its [the] critical miceller concentration.

14. **(Amended)** The method of [any of claims 1 to 13] Claim 1, [characterized in that] wherein said second detergent is alkyl-phosphorylcholine with a chain length of C10-C16 [is used as said second detergent].
15. **(Cancelled)**
16. **(Cancelled).**

ABSTRACT

REFOLDING OF MEMBRANE PROTEINS

In a method for production of membrane proteins or receptors folded into their native structure, first, proteins solubilized in a first detergent are provided. To induce folding of proteins into their native form, the first detergent is exchanged for a second detergent. Both for the first and for the second detergent, examples are shown.

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Refolding of Membrane Proteins

The present invention relates to a method for the production of proteins folded into their native or active structure, said protein being from the group comprising membrane proteins, in particular receptors, preferably from the family of G-protein-coupled receptors, as well as partial sequences, homologous sequences, mutated sequences and derived sequences of membrane proteins and receptors, with the steps:

- Providing of protein solubilized in a first detergent, and
- Exchange of said first detergent for a second detergent, which induces the folding of protein into its native or active form.

For membrane proteins, such a method is known from the article "Refolding of *Escherichia coli* produced membrane protein inclu-

A method for refolding of receptor protein is known from the article "Expression of an Olfactory Receptor in *Escherichia coli*: Purification, Reconstitution, and Ligand Binding" by Kiefer et al. in Biochemistry 35 (1996) 16077-16084.

For the membrane proteins Toc75 and LHCP, Rogl et al. describe a method in which N-Lauroylsarcosine is used as first detergent and Triton X-100® is used as second detergent. By exchanging the chaotrope for the mild detergent, refolding of the aggregated protein was induced.

In both cases, it could be shown that the aggregated protein first existing in the form of inclusion bodies was, first, solubilized in a denaturing detergent and, then, by the detergent exchange described, transformed into its active structure, which was verified by corresponding binding measurements.

There is a great scientific and commercial interest in membrane proteins, in particular in receptors in native or active form, since membrane proteins are components of all biological membranes and impart to the specificity of different cellular membranes, they are particularly responsible for the exchange of substances and signals.

The specific recognition of a chemical compound by the corresponding receptor has e.g. the consequence that the target cell changes its physiological state. That is why receptors are the most important target molecules for drugs, approximately 3/4 of all commercially available pharmaceuticals act on receptors, most of which, again, act on so-called G-protein-coupled receptors, which have in the human genome several hundreds of representatives.

For the development of specific antibodies, of drugs etc. it is, in view of the above, most desirable to have membrane proteins, in particular receptors in active or native structure available in large quantities. Since these proteins occur, in tissue, only in very small concentrations, it is necessary to use a system for recombinant over-expression of membrane proteins and receptors. For this purpose, on the one hand, in eukaryotic cells (cells of mammals or insects), functional protein can be produced, however, the systems are expensive, and the expression rates are low, which is also disadvantageous. Functional protein can be obtained via bacterial expression as well, the expression rate, however, is even lower than in eukaryotic expression.

In view of the above, the two publications mentioned at the outset describe methods, in which the protein is expressed in the inner part of the cell, where it, however, aggregates, and hence is not functionally available. The advantage of this

method is that very large quantities of protein can be produced, Kiefer et al. report that up to 10 % of cell protein and, thus, 100-10,000 times more protein than with other expression systems can be produced. The inclusion bodies produced in that way, which Rogl et al. have also reported about, must then first of all, be solubilized and, via the exchange of detergents already described at the outset, be transformed into their native or active structure.

Of course, commercial interest is not only directed to membrane proteins and receptors in their naturally existing sequence, rather, also partial sequences, homologous sequences, mutated sequences or derived sequences of membrane proteins and receptors are an object of this invention, as they allow, depending on functionality, not only insights into the structure of membrane proteins and receptors, but also a rational drug design.

In this context it should be mentioned that the DNA sequence of many receptors is known, such sequences are contained in the EMBL database. As these DNA sequences, in most cases, do not contain introns, the coding sequence can be produced by PCR from genomic DNA or by RT-PCR from mRNA. This DNA can then be cloned into a corresponding expression vector.

However, the structure of the translation product is unknown, so that providing proteins which are an object of the invention in sufficient quantity allows crystallization experiments etc. to further elucidate the structure.

It should also be mentioned that receptors expressed in eukaryotic and in bacteria can be distinguished by glycosylation. G-protein-coupled receptors, namely, possess on the N-terminus one or more glycosylation sites, which are modified in the endoplasmic reticulum or later in the Golgi apparatus with an

- Alkyl-N,N-dimethylglycine (alkyl = C8-C16)
- Alkylglycosides (alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides)
- Saccharide fatty acid ester (e.g. sucrosemonododecanoate)

- The object underlying the invention is in that way completely achieved.

The inventors of the submitted application have, namely, recognized that the low yield and the lacking reproducibility in the known methods is to be assigned to the second detergent. Surprisingly, the yields were, namely, distinctively higher and the results were more reproducible, if the second detergent was selected from the group mentioned above. Further, it has to be taken care that the second detergent, in its final concentration, is above the critical micellar concentration. This cmc-value hence reflects, in principle, the solubility of a detergent in water. Above the cmc-value, the concentration of solved detergent-monomers is constant.

The cmc-values of some detergents are described in the publication "Detergents: An Overview" J.M. Neugebauer in Methods in Enzymology 182 (1990), pages 239-253.

According to own measurements of the inventors, the cmc-values for alkyl-phosphorylcholine with an alkyl rest of C12, C13, C14 or C16 are 500, 150, 50 or 5 μM .

In this procedure, it is preferred if the protein is produced in form of inclusion bodies in a cell line transformed with an expression vector, which vector carries a gene coding for said protein, the protein being preferably part of a fusion protein and, before or after said exchange of detergents, is cleaved off from said fusion protein.

The expression of the DNA sequence coding for the protein according to the invention as fusion protein has, in comparison with the direct expression without carrier protein, the advantage that the carrier protein protects the protein which is desired, but, however, unknown to the expression system, against

degradation by proteases and may result in a higher expression level. In particular by using glutathione-S-transferase (GST) as carrier protein, the solubility of proteins being expressed in large quantities is increased in the host cell and isolation is facilitated. The carrier protein can, further, be used for purifying fusion proteins, if suitable antibodies are provided. The same applies for purification methods with affinity chromatography.

In this method, it is further preferred if the inclusion bodies are purified and solubilized by adding the first detergent, wherein the first detergent is selected from the group:

N-Lauroylsarcosine, Dodecylsulfate, other charged detergents or urea or guanidiniumchloride in combination with charged or uncharged detergents.

It is important in this connection that the conditions to bring the protein in solution are denaturing, so that they do not allow a formulation of the native structure.

In this method, it is altogether preferred if the second detergent is present in a folding buffer with mixed lipid/detergent micelles, wherein the folding buffer contains preferably the second detergent and phospholipid from a natural source, preferably a lipid extract of tissue, in which the protein occurs naturally.

In this method, it is advantageous that, in comparison with the use of pure detergent micelles, the yield of native protein can even be improved. The lipid extract of the tissue, in which the receptor occurs naturally, can also be simulated by using lipids with a similar composition or by mixing same.

With reference to the exchange of detergents it is preferred, if same is done by a dialysis or ultrafiltration method or by chromatographic methods or by diluting said solubilized protein in a buffer which contains said second detergent.

The methods described insofar concerning the exchange of detergents are exchangeable amongst each other and offer, each for itself, specific advantages with reference to the handling, the duration of the method and the reachable yield.

After said exchange of detergents, at least one conserved disulfide bridge must be formed in the protein, preferably by adding a mixture of oxidized and reduced glutathione.

The folded protein can further be incorporated in proteoliposomes, which are artificially produced vesicles and represent a functional unit. With the help of these deliberately produced proteoliposomes, certain processes on the membrane proteins/receptors can be selectively investigated.

In view of the above, the present invention relates further to proteoliposomes with protein produced according to the method mentioned above.

Furthermore, the present invention relates to the use of a detergent for the production of proteins folded into their native or active structure of the kind described above, whereby the detergent is selected from the group:

- Alkyl-N,N-dimethylglycine (alkyl = C₈-C₁₆)

- Alkylglycosides (alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides)
- Saccharide fatty acid ester (e.g. sucrosemonododecanoate)
- Alkylthioglycosides (alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides with S- instead of O-glycosidic bond)
- Bile acids (cholate, deoxycholate) and derivatives (e.g. CHAPS, CHAPS0)
- Glucamides (MEGA-8 to -10, HEGA)
- Lecithins and lysolecithins (e.g. DHPC, C12-lysolecithin)
- Alkyl-Phosphorylcholine (Alkyl = C10 - C16).

Further advantages can be taken from the following description of preferred embodiments.

It is to be understood that the features mentioned above and those yet to be explained below can be used not only in the respective combinations indicated, but also in other combinations or in isolation, without leaving the scope of the present invention.

The invention will be explained in more detail in the description below.

Example 1: Production of an Expression Vector with cDNA
 for Receptor Protein

DNA sequences for several receptor proteins and also membrane proteins are in the EMBL database, in most cases, they do not have introns. With the help of primers, the required DNA can be produced via PCR from genomic DNA or via RT-PCR from mRNA.

This DNA is then cloned into an expression vector, which was constructed for the expression of a fusion protein. The carrier protein can be e.g. glutathione-S-transferase (GST), as described in the article by Kiefer et al. mentioned above, wherein a fusion protein was produced from the receptor OR5 and GST. The expression vector is transformed into a cell line which expresses the fusion protein. The protein is, in this procedure, not incorporated into the membrane, but exists at least partly aggregated in form of inclusion bodies in cytoplasm and is, thus, not correctly folded.

Example 2: Isolation of Expressed Protein

The cDNA of one of the following receptors is, in-frame, inserted into the vector pGEX2a-c-His: AD adenosine receptor from the shark *Squalus acanthias*, human beta-2-adrenergic receptor, human neuropeptide YY1 receptor, human neuropeptide YY2 receptor, human melanocortine-1 receptor, human oxytocin receptor. This vector contains downstream of the Tac-promotor the sequence encoding glutathione-S-transferase and a subsequent thrombin cleavage site, followed by a polylinker sequence and, finally, six histidine codons and a stop codon.

The vectors are transformed into the E. coli strain BL21. The protein expression is induced by adding IPTG, and the cells are harvested after further three hours. After lysozyme treatment

and homogenization by sonication, the membranes and inclusion bodies are separated from the soluble proteins by centrifugation.

Example 3: Solubilization of the Protein and Detergent exchange by Column Chromatographic

The inclusion bodies are solubilized by adding 1.5 % N-Lauroylsarcosine at 0°C and diluted to fivefold volume with buffer (0.1 % Alkyl(C14)phosphorylcholine). Thrombin is added to this solution and incubation is performed for 16 hours at 20°C to separate the receptor from GST. After that, insoluble cell parts are isolated by centrifugation.

The supernatant is loaded onto Ni-NTA-Agarose (Qiagen) and incubated for one hour at 4°C, wherein the receptor binds to the nickel matrix. Thereafter, the nickel material is applied to a column and - to exchange detergent - washed with buffer, containing 0.01 % Alkyl(C14)phosphorylcholine as second detergent. In that way, the N-Lauroylsarcosine (first detergent) and contaminating proteins are removed.

Example 4: Reconstitution of the Protein

For the reconstitution, a lipid mixture consisting of 70 % 1-Palmitoyl-2-oleoylphosphatidylcholine and 30 % 1-Palmitoyl-2-oleoylphosphatidylglycerol is dissolved in chloroform together with the double (w:w) amount of dodecylmaltoside and the solvent is removed under vacuum. The purified protein obtained in example 3 is added and incubation is performed for at least one hour. The detergent is removed via a polystyrene column (Calbiosorb from Calbiochem), whereupon liposomes with incorporated receptor are formed (proteoliposomes).

By measuring ligand binding it could be shown that the receptor is present in native structure.

Example 5: Detergent exchange with Lipid/Detergent Micelles

The following stock solutions were prepared:

- Cholesterol, Sigma C8667, 100 mg/ml in CHCl_3
- Sheep brain phospholipid, Sigma P4264, 100 mg/ml in CHCl_3
- Soybean lecithin, Sigma P3644, 100 mg/ml in CHCl_3
- 100 mg of Alkyl(C16)-phosphorylcholine in 50 ml flasks, dissolved in 1-2 ml CHCl_3 ; adding 28 μg of cholesterol stock solution, 32 μl of sheep brain phospholipid stock solution, 40 μl of soybean lecithin stock solution; evaporation of CHCl_3 and drying for at least 30 minutes at less than 15 mbar; adding 1 ml of water to obtain a clear solution (detergent stock solution, 100 mg/ml)
- thrombin, Sigma T4648, 1000 u/ml in H_2O , stored at -20°C
- sarcosyl: 10 % N-Lauroylsarcosine in H_2O , autoclaved
- 10 x PBS: 200 mM sodium phosphate, 1.5 M NaCl, pH 7.0

2 ml of 3 % sarcosyl are resuspended in PBS and stored on ice. 2 ml of inclusion bodies obtained in example 2 are added and mixed and treated for one minute with sonication. Immediately afterwards, 16 ml of 0.1 % detergent stock solution in PBS are added.

At this point already, an exchange of detergents takes place, sarcosyl is diluted below the cmc-value, while the end concentration of Alkyl(C16)phosphorylcholine is above the cmc-value.

After adding 15 u of thrombin, the solution is kept over night at 20°C in order to cleave the fusion protein.

After that, the solution is centrifuged for 30 minutes at 4°C with 40,000 rpm and the supernatant is removed.

To the supernatant, 20 mM imidazol of a 1 M stock solution (pH 7.0) are added. This solution is added to an appropriately equilibrated column resin Ni-NTA superflow (Qiagen) and is moderately rotated in a cold room (4-8°C) for one hour to prevent the material from settling. In that way, the receptor protein binds to the column resin.

After that, the column resin is centrifuged for one minute at 2000 rpm, and the supernatant is removed to such an extent that the remaining supernatant corresponds to the bed volume. Ni-NTA agarose is taken up and loaded onto a column. At a flow rate of 2 ml/min, washing is performed with 40 ml of a 0.01 % detergent stock solution in PBS, whereby a further exchange of detergents is performed with consideration of cmc-values.

The column is then eluted with 10 ml of PBS/0.01 % detergent stock solution/0.3 M imidazole, and the fractions absorbing at 280 nm are collected.

Thereafter, a dialysis against 2 l of PBS is performed for four hours, as well as adding of 1 mM of GSH/0.1 mM GSSG from a 100 x stock solution in water.

- Alkyl-N,N-dimethylglycine (alkyl = C8-C16)
- Alkylglycosides (alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides)
- Saccharide fatty acid ester (e.g. sucrosemonododecanoate)
- Alkylthioglycosides (alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides with S- instead of O-glycosidic bond)
- Bile acids (cholate, deoxycholate) and derivatives (e.g. CHAPS, CHAPS0)
- Glucamides (MEGA-8 to -10, HEGA)
- Lecithins and lysolecithins (e.g. DHPC, C12-lysolecithin)
- Alkyl-Phosphorylcholine (Alkyl = C10 - C16)

10. The method of any of claims 1 to 9, characterized in that said protein is part of a fusion protein and, before or after said exchange of detergents, is cleaved off from said fusion protein.
11. The method of claim 9, characterized in that said inclusion bodies are purified and, by adding said first detergent, solubilized.
12. The method of any of claims 1 to 11, characterized in that said first detergent is selected from the group N-Lauroylsarcosine, dodecylsulfate, other charged detergents or urea or guanidiniumchloride in combination with charged or uncharged detergents.
13. The method of any of claims 1 to 12, characterized in that said second detergent is used above the critical micellar concentration.
14. The method of any of claims 1 to 13, characterized in that alkyl-phosphorylcholine with a chain length of C10-C16 is used as said second detergent.
15. Proteoliposomes with protein produced according to the method of any of claims 1 to 14.
16. The use of a detergent for production of proteins folded into their native or active structure, said proteins being from the group comprising membrane proteins, in particular receptors, preferably from the family of G-protein-coupled receptors, as well as partial sequences, homologous sequences, mutated sequences and derived sequences of membrane proteins and receptors, characterized in that said detergent is selected from the group:

- Alkyl-N,N-dimethylglycine (alkyl = C8-C16)
- Alkylglycosides (alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides)
- Saccharide fatty acid ester (e.g. sucrosemonododecanoate)
- Alkylthioglycosides (alkyl = C5-C12, also branched-chained or cyclic alkyl rests, glycoside = all mono- and disaccharides with S- instead of O-glycosidic bond)
- Bile acids (cholate, deoxycholate) and derivatives (e.g. CHAPS, CHAPS0)
- Glucamides (MEGA-8 to -10, HEGA)
- Lecithins and lysolecithins (e.g. DHPC, C12-lysolecithin)
- Alkyl-Phosphorylcholine (Alkyl = C10 - C16).

Abstract

In a method for production of membrane proteins or receptors folded into their native structure, first, proteins solubilized in a first detergent are provided. To induce folding of proteins into their native form, the first detergent is exchanged for a second detergent. Both for the first and for the second detergent, examples are shown.

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DECLARATION AND POWER OF ATTORNEY - USA PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled REFOLDING OF MEMBRANE PROTEINS the specification of which was described and claimed in PCT International Application No. EP00/07763 filed on August 10, 2000.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above;

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56;

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent, design or inventor's certificate or any PCT international application(s) listed below and have also identified below any foreign application(s) for patent, design or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed for the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

COUNTRY (OR INDICATE IF PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 37 U.S.C. § 119
German	19939246.3	19, 08, 1999	• YES NO o

POWER OF ATTORNEY: I hereby appoint the registrants of Knobbe, Martens, Olson & Bear, LLP, 620 Newport Center Drive, Sixteenth Floor, Newport Beach, California 92660, Telephone (949) 760-0404, Customer No. 20,995.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor: **Hans KIEFER**

Inventor's signature Hans Kiefer Day 22 Month April Year 2002

Residence (city and country): Stuttgart, Germany **DEX**

Citizenship: German

Post Office Address: Riedgrasweg 70, D-70599 Stuttgart, Germany

2.00 Full name of second inventor: Klaus MAIER

Inventor's signature Klaus Maier Day 22 Month April Year 2002

Residence (city and country): Stuttgart, Germany DEX

Citizenship: German

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Customer No. 20,995

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ASSIGNMENT OF RIGHT OF ASSIGNEE TO TAKE ACTION
AND
REVOCATION AND POWER OF ATTORNEY

To the Commissioner of Patents and Trademarks:

The undersigned is empowered to act on behalf of the assignee indicated below (the "Assignee"). The original assignment of the U.S. Application No. 10/069,433, filed February 19, 2002, for Letters Patent for the invention in REFOLDING OF MEMBRANE PROTEINS from the inventors to the Assignee is being submitted herewith for recordation by the Assignment Branch. A true copy of this Assignment is attached hereto. This Assignment represents the entire chain of title of this invention from the Inventor(s) to the Assignee. I have reviewed this Assignment, and to the best of the Assignee's knowledge and belief, the Assignee is the owner of the entire right, title, and interest in the above-referenced application.

I declare that all statements made herein of my own knowledge are true, and that all statements made upon information and belief are believed to be true, and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that willful, false statements may jeopardize the validity of the application, or any patent issuing thereon.

The undersigned hereby revokes any previous powers of attorney in the subject application, and hereby appoints the registrants of Knobbe, Martens, Olson & Bear, LLP, 620 Newport Center Drive, Sixteenth Floor, Newport Beach, California 92660, Telephone (949) 760-0404, **Customer No. 20,995**, as its attorneys with full power of substitution and revocation to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected herewith. This appointment is to be to the exclusion of the inventor(s) and his attorney(s) in accordance with the provisions of 37 C.F.R. § 3.71.

Please use **Customer No. 20,995** for all communications.

Assignee: M-phasys GmbH

By: Wolfgang Arndt

Printed Name: Wolfgang Arndt

Title: Managing Director

Address: Vor dem Kreuzberg 17, D-72070 Tübingen
GERMANY

April, 22, 2002

Dated: _____

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PATENT

ASSIGNMENT

WHEREAS, I, Hans Kiefer, a German citizen, residing at Riedgrasweg 70, D-70599 Stuttgart, Germany, and Klaus Maier, a German citizen, residing at Paracelsusstrasse 79, D-70599 Stuttgart, Germany, hereinafter referred to as Assignor (collectively if more than one inventor is listed above), have invented certain new and useful improvements in REFOLDING OF MEMBRANE PROTEINS, the specification of which was described and claimed in PCT International Application No. EP00/07763, filed on August 10, 2000.

AND WHEREAS, M-phasys GmbH, with its principal place of business at Vor dem Kreuzberg 17, D-72070 Tübingen, Germany (hereinafter referred to as Assignee) desires to acquire the entire right, title, and interest in and to the said improvements with respect to the United States of America, its territories and possessions.

NOW, THEREFORE, for good and valuable consideration, the receipt and sufficiency of which is hereby acknowledged, Assignor hereby acknowledges that it has sold, assigned, transferred and set over, and by these presents does hereby sell, assign, transfer and set over, unto Assignee, its successors, legal representatives and assigns, the entire right, title, and interest in the United States of America, and its territories and possessions in, to and under said improvements, and any Patent Applications in the United States of America and all divisions, renewals and continuations thereof, and all Patents of the United States of America which may be granted thereon and all reissues and extensions thereof, and all rights of priority under International Conventions; and Assignor hereby authorizes and requests the Commissioner of Patents of the United States of America to issue all Patents for said improvements to Assignee, its successors, legal representatives and assigns, in accordance with the terms of this instrument.

AND ASSIGNOR HEREBY covenants and agrees that it will communicate to Assignee, its successors, legal representatives and assigns, any facts known to it respecting said improvements, and testify in any legal proceeding, sign all lawful papers, execute all divisional, continuing and reissue applications, make all rightful oaths and generally do everything possible to aid Assignee, its successors, legal representatives and assigns, to obtain and enforce proper patent protection for said improvements in the United States of America.

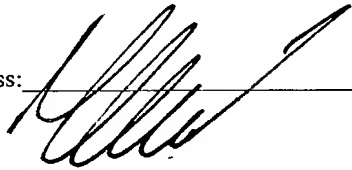
IN TESTIMONY WHEREOF, Assignor intending to be legally bound has hereunto affixed its signature.

This 22 day of April, 2002



Signature of Hans KIEFER

Witness:



This 22 day of April, 2002



Signature of Klaus MAIER

Witness:

